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Authors
Miller, BR
Cumsky, MG

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Intramitochondrial Sorting of the Precursor to Yeast Cytochrome *c* Oxidase Subunit Va

Brian R. Miller and Michael G. Cumsky

Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92717

Abstract. We have continued our studies on the import pathway of the precursor to yeast cytochrome *c* oxidase subunit Va (pVa), a mitochondrial inner membrane protein. Previous work on this precursor demonstrated that import of pVa is unusually efficient, and that inner membrane localization is directed by a membrane-spanning domain in the COOH-terminal third of the protein. Here we report the results of studies aimed at analyzing the intramitochondrial sorting of pVa, as well as the role played by ancillary factors in import and localization of the precursor. We found that pVa was efficiently imported and correctly sorted in mitochondria prepared from yeast strains defective in the function of either mitochondrial heat shock protein (hsp)60 or hsp70. Under identical conditions the import and sorting of another mitochondrial protein, the precursor to the β subunit of the F_1ATPase, was completely defective. Consistent with previous results demonstrating that the subunit Va precursor is loosely folded, we found that pVa could be efficiently imported into mitochondria after translation in wheat germ extracts. This result suggests that normal levels of extramitochondrial hsp70 are also not required for import of the protein. The results of this study enhance our understanding of the mechanism by which pVa is routed to the mitochondrial inner membrane. They suggest that while the NH_2 terminus of pVa is exposed to the matrix and processed by the matrix metalloprotease, the protein remains anchored to the inner membrane before being assembled into a functional holoenzyme complex.

THE vast majority of the proteins that constitute a functional mitochondrion are the products of nuclear genes that are targeted to the organelle after translation in the cytosol. Not only must these polypeptides be delivered to the mitochondrion, they must also be sorted to the correct location within the organelle. The import and sorting of precursor proteins, therefore, represents a fundamental problem in mitochondrial biogenesis.

Mitochondria contain four distinct compartments: the outer membrane, inner membrane, matrix, and intermembrane space. In recent years the delivery of polypeptides to the matrix has been extensively characterized (for current reviews see Neupert et al., 1990; Pfanner and Neupert, 1990; Glick and Schatz, 1991; Glick et al., 1992*b*). During or after translation in the cytosol, precursor proteins are maintained in an "import-competent" conformation by heat shock protein (hsp)[1]70 and (possibly) by other factors in the cytosol (Deshaies et al., 1988, Murakami et al., 1988). Import is mediated by a positively charged NH_2-terminal extension called a leader peptide or presequence (Hurt et al., 1984, 1985; Horwich et al., 1985), which is present on the majority of nuclear-encoded mitochondrial precursor proteins. In the presence of a membrane potential ($\Delta\psi$), and after interaction with proteinaceous receptor-like components at the mitochondrial surface, the translocation process is thought to begin by electrophoresis of the presequence into the organelle at sites of contact between distinct translocation machineries at the outer and inner membranes (Glick et al., 1991; Martin et al., 1991; Pfanner et al., 1992). After transfer of the presequence across the inner membrane, the sequential action of the matrix proteins mitochondrial hsp70 (mhsp70) and hsp60 facilitates translocation and folding of the imported precursor in an ATP-dependant manner (Cheng et al., 1989; Ostermann et al., 1989; Kang et al., 1990; Mahlke et al., 1990; Ostermann et al., 1990; Scherer, et al., 1990; Manning-Krieg et al., 1991). At some point during or after translocation, the presequence is removed by a metalloprotease located in the matrix (Hawlitshek et al., 1988; Jensen and Yaffe, 1988).

In contrast to matrix localization, sorting to the other mitochondrial compartments is only beginning to be understood. The most extensive studies have been performed on precursors destined for the intermembrane space (including those partly associated with the external face of the inner

B. R. Miller's present address is Department of Biology, University of California, San Diego, La Jolla, CA 92093.

1. *Abbreviations used in this paper:* AAC, ADP/ATP carrier; chsp70, cytosolic hsp70; hsp, heat shock protein; IMSS, inner membrane sorting signal; mhsp70, mitochondrial hsp70; NEM, N-ethylmaleimide; pVa, presubunit Va; Val, valinomycin.

membrane). Many of these proteins contain bipartite presequences composed of a typical matrix targeting signal (above) and a hydrophobic sorting signal (Hurt and van Loon, 1986; Hartl et al., 1987). Recently, two distinct translocation pathways to the intermembrane space have been proposed (reviewed in Hartl and Neupert, 1990; Glick and Schatz, 1991; Glick et al., 1992*a*,*b*). The first, generally referred to as the "stop-transfer" or "nonconservative" model, was initially based upon the identification of translocation intermediates that spanned the inner membrane in a manner that exposed the NH_2 terminus to the matrix and the majority of the mature domain to the intermembrane space (Daum et al., 1982; van Loon and Schatz, 1987). Further support for the model came from the finding that the matrix-targeting portion of a bipartite presequence directed an attached passenger protein to the matrix, while the entire presequence (containing the hydrophobic sequence) directed the same passenger to the intermembrane space (van Loon et al., 1986). According to the model, the hydrophobic sorting sequence acts to block the complete transfer of the protein across the inner membrane, making the process analogous to the mechanism by which proteins are inserted into the membrane of the ER (Blobel, 1980). The matrix targeting portion of the presequence is cleaved by the matrix protease, while a second protease located in the intermembrane space generates the mature protein (van Loon and Schatz, 1987; Schneider et al., 1991).

The second model, referred to as the "reexport" or "conservative sorting" hypothesis, proposes that precursor proteins destined for the intermembrane space pass completely into the matrix before being reexported back across the inner membrane. The key observations upon which this model was formed were that partially processed translocation intermediates could be identified in the matrix (Hartl et al., 1986, 1987), and that the function of hsp60 was required for proper localization of several precursors (Cheng et al., 1989; Hartl and Neupert, 1990; Mahlke et al., 1990). According to this model, the sorting sequence functions to redirect proteins, which are still unfolded and associated with hsp60, back into and/or across the inner membrane. This process, therefore, is analogous to the mechanism by which proteins are exported in bacteria (Hartl and Neupert, 1990).

At the present time it is unclear which sorting pathway is used by the majority of the proteins of the inner membrane. Furthermore, because the inner membrane is composed of integral as well as peripheral proteins located on both the matrix and intermembrane face of the bilayer, it is reasonable to speculate that this diverse group of polypeptides may follow several different sorting pathways. Thus far, the available data bear out this speculation. The precursor to the ADP/ATP carrier (AAC), an integral inner membrane protein that does not contain an NH_2-terminal presequence, is sorted in a nonconservative manner; it does not completely cross the inner membrane or require the function of hsp60 for proper localization (Pfanner and Neupert, 1987; Mahlke et al., 1990). In contrast, the precursor to the Rieske Fe/S protein, a peripheral protein located on the intermembrane face of the inner membrane, appears to be sorted conservatively (Hartl et al., 1986; van Loon and Schatz, 1987). It is first translocated into the matrix where the presequence is cleaved twice; it is then reexported to the external face of the inner membrane in an hsp60-dependant fashion (Cheng et al., 1989; Hartl and Neupert, 1990). The conservative sorting of the Fe/S protein is presumably mediated by sequences within the mature portion of the polypeptide (Hartl and Neupert, 1990).

Over the last several years our laboratory has been elucidating targeting pathways to the mitochondrial inner membrane. In particular, we have focused on the precursor to subunit Va of yeast cytochrome *c* oxidase, a small integral inner membrane protein (Cumsky et al., 1985, 1987). We have shown that import of this precursor is unusually efficient and requires only a minimal presequence (Glaser et al., 1988, 1990). In addition, import of presubunit Va (pVa) is independent of protease-sensitive surface receptors, requires only low levels of ATP, and is not affected by temperature (Miller and Cumsky, 1991). We have also shown that all of the information required for intramitochondrial localization of pVa is contained in a hydrophobic domain near the COOH terminus of the mature polypeptide. Removal of this localization domain (referred to as the inner membrane sorting signal, IMSS) causes the protein to be mislocalized to the mitochondrial matrix (Glaser et al., 1990).

The lack of an observable matrix-bound intermediate for subunit Va, and the observation that it was correctly sorted to the inner membrane at low temperature, led us to hypothesize that the IMSS acted as a stop-transfer sequence which prevented complete translocation across the inner membrane. However, the data could not eliminate the possibility that pVa was completely translocated and the matrix-localized intermediate rapidly exported. Indeed, the rapid import kinetics of pVa, even at low temperature, has made the detection of any translocation intermediate difficult (Miller and Cumsky, 1991).

In the present report, we have continued our examination of the sorting pathway for subunit Va. We show that neither the import nor the sorting of pVa require the function of hsp60 in the matrix. We also show that import and sorting of pVa occur normally when mitochondrial hsp70 function is severely impaired or altogether lacking. Finally, we present evidence that pVa import is largely independent of soluble factors from the cytosol, including hsp70. The results presented here, in conjunction with additional work on the subunit Va sorting signal, are most consistent with a stop-transfer model for delivery of pVa to the mitochondrial inner membrane.

Materials and Methods

In Vitro Transcription and Translation

In vitro transcription and translation of both pVa and pre-$F_1\beta$ (p$F_1\beta$, the precursor to the β subunit of yeast F_1 ATPase) in rabbit reticulocyte lysates has been previously described (Miller and Cumsky, 1991). Translation of both precursors in wheat germ extracts (Promega Biotec, Madison, WI) was performed as recommended by the manufacturer in the presence of [^{35}S]methionine. As with translation reactions performed using reticulocyte lysates, precursors synthesized in wheat germ extracts were centrifuged at 16,000 *g* to remove aggregates. No apparent differences in import efficiency was detected between freshly translated precursors or translation reactions stored at −70°C.

In Vitro Import Reactions

Wild-type mitochondria were prepared from the *S. cerevisiae* strain D273-10B (ATCC 24657) which was grown to mid-log phase in YPGE medium

(Sherman et al., 1986). The isolation procedure has been described previously (Miller and Cumsky, 1991). Mitochondria defective in the matrix chaperonin hsp60 were prepared from the *mif4 S. cerevisiae* strain α143 (Cheng et al., 1989) with the following modifications of the standard procedure. Cells were grown in YPGE at 22–24°C until they reached mid-log phase, then shifted to 37°C for 3.5 h. The cultures were chilled rapidly on ice and the cells harvested. They were then washed twice in ice cold 1.2 M sorbitol/10 mM K^+ Hepes, pH 7.2, before spheroplasting. Mitochondria defective in the *SSC1* isoform of hsp70 were prepared from the *S. cerevisiae* strain BC100(*ssc1-2*) (Kang et al., 1990) in the same manner as for wild type except that cultures were grown and cells spheroplasted at 22–24°C.

In vitro import reactions (100 μl vol) were performed in TRB buffer (250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 20 mM 3-[N-morpholino]propanesulfonic acid [MOPS], pH 7.2, 3% BSA, 1 mM ATP, and 10 mM of each K^+-succinate and K^+-malate) as previously reported (Miller and Cumsky, 1991) except that the 10 mM K^+-succinate/K^+-malate was replaced with 2 mM NADH. Each reaction contained 50 μg mitochondrial protein and 5–20,000 cpm of precursor. Unless otherwise indicated, the import reactions were incubated for 5 min at room temperature (22–24°C) before they were terminated by the addition of valinomycin (Val) to 10 μg/ml and placed on ice. Importantly, when incubated for these short time intervals, the amount of protease-protected mature species observed also provides a reliable measure of import efficiency, since the rate of pVa import has previously been determined (Miller and Cumsky, 1991). Significant alterations in import kinetics are detectable in reactions of 5 min of less (Miller and Cumsky, 1991). Unless otherwise indicated, protease sensitivity was determined by the addition of proteinase K to 100 μg/ml for 20 min at 0°C and terminated by the addition of PMSF to 1 mM. The mitochondria were collected by centrifugation and processed for SDS-PAGE as described (Glaser and Cumsky, 1990*a*).

Import reactions using precursor proteins translated in wheat germ extracts required the following modifications of the standard assay. To reduce the amount of cytosolic factor(s) that co-purify with the mitochondria, the organelles were washed with SEMK (250 mM sucrose, 1 mM EDTA, 20 mM MOPS, 200 mM KCl, pH 7.2) for 15 min at room temperature. Also, because of the low translation efficiency of pVa in wheat germ extracts (Miller, B. R., unpublished results) larger volumes (up to 5 μl) of the translation reaction were required in import assays. For $pF_1\beta$, which was translated efficiently, translation extracts were diluted with cold wheat germ extract to achieve the same specific activity as that of pVa. Control experiments indicated that the addition of 5 μl of undiluted $pF_1\beta$ in wheat germ extract had no apparent effect on the relative import efficiency of the protein.

Mitochondria prepared from the *ssc1-2* strain were treated essentially as described (Kang et al., 1990) except that the mitochondria were reisolated in TRB after the heat shock at 37°C. Import reactions were incubated with precursor proteins in reticulocyte lysate at room temperature for 5 min, then terminated by the addition of Val to 10 μg/ml. Protease sensitivity was determined as described above.

Mitoplasts were prepared as described previously (Glaser and Cumsky, 1990*b*; Glaser et al., 1990). The formation of the m′ form of subunit Va (Miller and Cumsky, 1991) is a sensitive indicator of the integrity of the mitochondrial outer membrane and is diagnostic of correct sorting of subunit Va to the inner mitochondrial membrane (see Results).

Chloroform Extraction of Mitochondria

To determine whether the β subunit was assembled into the F_1 portion of ATPase, the following modifications were made to the standard import assay. Import reactions programmed with $pF_1\beta$ were incubated for 20 min at room temperature, terminated with valinomycin, and treated with proteinase K (100 μg/ml) for 5 min at room temperature. The digestions were stopped by the addition of PMSF to a final concentration of 1 mM and the mitochondria reisolated by centrifugation at 16,000 *g* for 10 min. They were then resuspended in 100 μl of FEB (10 mM Tris-SO_4, pH 7.6, 1 mM ATP, 10 mM $MgSO_4$, 0.1 mM PMSF, 20 μg/ml α_2-macroglobulin). The samples were placed on dry ice for 10 min, allowed to thaw to room temperature, and extracted with 50 μl of chloroform by vortexing for 2 min. The samples were then centrifuged for 2 min at room temperature (16,000 *g*) and the upper aqueous phase transferred to a fresh tube. After TCA (aqueous phase) or methanol (interface and organic phase) precipitation, the pellets were resuspended in SDS sample buffer and analyzed by SDS-PAGE.

Triton X-100 Extraction of hsp60

Aggregated hsp60 from the α143 (*mif4*) strain was distinguished from the native form by its solubility in Triton X-100 (Cheng et al., 1989). Mitochondria (100 μg) isolated from either wild-type cells (grown at 30°C) or from heat-shocked (37°C, 3.5 h) α143 cells were suspended 100 μl Triton buffer (10 mM Hepes, 1 mM EDTA, 1% Triton X-100, pH 7.2) and incubated at 0°C for 5 min. To determine the total amount of hsp60 in each sample, 50 μl aliquots were removed and diluted into 2 × SDS sample buffer. The remainder of each sample was then centrifuged at 16,000 *g* for 5 min at room temperature. The supernatants were removed to a fresh tube and diluted into 50 μl 2 × SDS sample buffer. The pellets were resuspended in 100 μl 1 × SDS sample buffer. The samples were then processed and analyzed by SDS-PAGE and immune blotting using anti-hsp60 antiserum.

NEM Pretreatment of Mitochondria

Mitochondria were resuspended in SEM buffer to 10 mg/ml and *N*-ethylmaleimide (NEM) added from a freshly prepared aqueous solution to a final concentration of 5 mM. After 10 min at room temperature, DTT was added to a final concentration of 25 mM to quench unreacted NEM. The mitochondria were then diluted into TRB and used for import assays. Control mitochondria were treated with an equivalent amount of NEM that had been prereacted with DTT. Import assays were performed in the standard manner as described above.

Miscellaneous Procedures

Protein determinations, SDS-PAGE, immune blotting, fluorography, and densitometric analysis of fluorographed bands were performed as described previously (Glaser and Cumsky, 1990*a*; Miller and Cumsky, 1991).

Results

Import and Sorting of pVa in Mitochondria Lacking a Functional hsp60 Complex in the Mitochondrial Matrix

The role of hsp60 as a catalyst in the folding and assembly of mitochondrial proteins has been clearly established (Cheng et al., 1989; Ostermann et al., 1989; Mahlke et al., 1990; Manning-Krieg et al., 1991). Current models predict, and the available data suggest, that hsp60 is essential for the proper sorting and assembly of mitochondrial precursor proteins that are completely translocated into the matrix (conservative sorting; Hartl and Neupert, 1990). In an elegant study, Ostermann et al. demonstrated that hsp60 function is affected by temperature; lowering the temperature from 30 to 10°C slowed the rate of hsp60-mediated folding several-fold within a 6-min import reaction (Ostermann et al., 1989). In an earlier work, we demonstrated that pVa import is unaffected by temperature; the rate of pVa import and sorting is essentially the same at both 0 and 30°C (Miller and Cumsky, 1991). Thus, our results argue against hsp60 playing an obligatory role in the import pathway of pVa.

To assess directly the role of hsp60 in the import and sorting of pVa, we first attempted to identify a subunit Va-hsp60 complex in mitochondria that had been depleted of ATP by treatment with apyrase. No such complex was found (data not shown). Therefore, we next analyzed import of pVa into mitochondria isolated from the yeast strain α143. This strain carries a temperature-sensitive form of hsp60, the product of the *MIF4* gene (Cheng et al., 1989). Cultures of the *mif4* (α143) strain were grown to mid-log phase at 22–24°C, shifted to the nonpermissive temperature (37°C) for 3.5 h, and mitochondria isolated. These mitochondria behaved identically to mitochondria isolated from a wild-type strain with respect to their ability to import and process pVa to a protease-protected mature form (Fig. 1 *A*, lanes *2* and *4*). Import into *mif4* mitochondria required an energized inner membrane ($\Delta\psi$, data not shown) and occurred at rates nearly

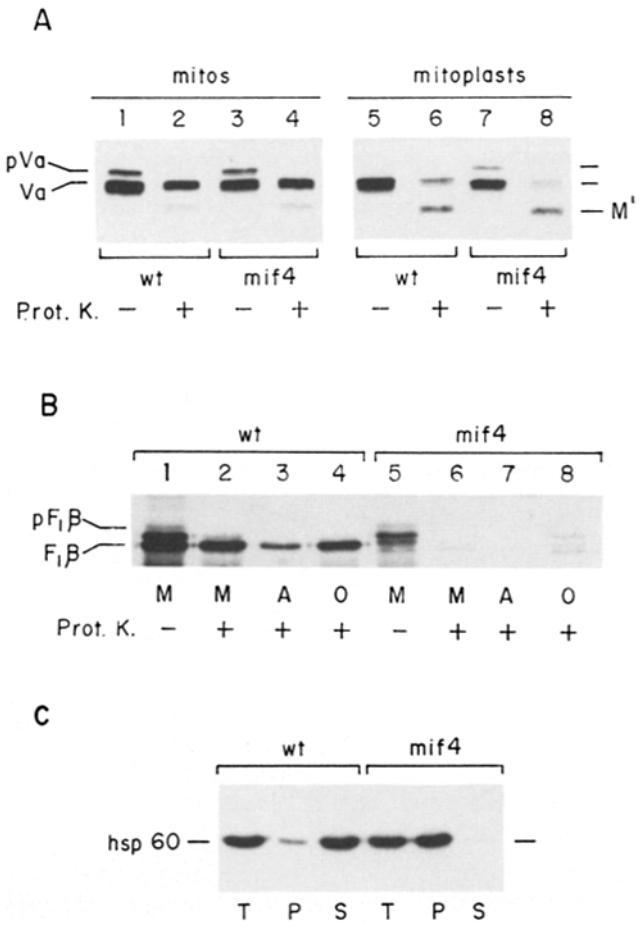

Figure 1. Import and sorting of yeast pVa does not require hsp60 function in the matrix. (*A*) Mitochondria were isolated from cultures of either the wild-type (lanes *1*, *2*, *5*, and *6*) yeast strain grown at 30°C or those from the *mif4* mutant strain (lanes *3*, *4*, *7*, and *8*) grown at room temperature and subsequently shifted to 37°C for 3.5 h to inactivate the mitochondrial hsp60. Where indicated (lanes *5–8*), the isolated mitochondria were converted to mitoplasts by osmotic shock (see Materials and Methods). Each import reaction contained 50 μg of the appropriate mitochondria or mitoplasts in 100 μl TRB and 15,000 cpm radiolabeled pVa. The reactions were performed in duplicate and incubated at room temperature for 5 min. After the addition of valinomycin (Val) to terminate the reactions, one of each of the duplicate reactions was treated with proteinase K (*Prot. K*; see Materials and Methods). The mitochondria or mitoplasts were then collected, processed for, and analyzed by SDS-PAGE and fluorography. The position of the precursor (*pVa*), mature (*Va*) and m′ (*M′*) forms of subunit Va are indicated. (*B*) Import and assembly of $pF_1\beta$ is defective in *mif4* mitochondria. Wild-type (lanes *1–4*) and *mif4* (lanes *5–8*) mitochondria were from the same preparation used in *A*. Import reactions were performed essentially as in *A* except that the reactions were done in batch. Each reaction contained 150 μg wild-type or *mif4* mitochondria in 300 μl TRB; the amount of radiolabeled $pF_1\beta$ added to each was 300,000 cpm. After incubation at room temperature for 20 min, both reactions were terminated with Val. 100 μl aliquots were removed and the mitochondria then reisolated by centrifugation and processed for SDS-PAGE (lanes *1* and *5*). The remainder of each reaction was digested with proteinase K (100 μg/ml) for 5 min at room temperature, stopped with PMSF, and another 100-μl aliquot removed and processed for SDS-PAGE (lanes *2* and *6*). The remaining mitochondria from each reaction were reisolated by a brief centrifugation at room temperature and resuspended in 100 μl of FEB (see Materials and Methods). After freezing on dry ice for 10 min, the samples were thawed to room temperature, extracted with chloroform (see Materials and Methods), and separated into aqueous (*A*; lanes *3* and *7*) and organic (*O*; lanes *4* and *8*) phases. The samples were precipitated with either TCA (aqueous phase) or methanol (organic phase and interface) and processed for SDS-PAGE. All samples were then analyzed by SDS-PAGE and fluorography. The position of the precursor ($pF_1\beta$) and mature forms of $F_1\beta$ are indicated. (*C*) Solubility of wild-type and mutant hsp60 proteins in Triton X-100. 100 μg of either wild type or *mif4* mitochondria prepared in *A* were dissolved in 100 μl of Triton buffer (see Materials and Methods) and placed on ice for 5 min. 50 μl of each sample was diluted into 50 μl 2 × SDS sample buffer for determination of the total amount of hsp60 protein. The remainder of each was centrifuged for 5 min at room temperature (16,000 *g*). The supernatants were removed and diluted into 50 μl of 2 × SDS sample buffer. The pellet was resuspended in 100 μl 1 × SDS sample buffer. 10 μl of each of the total (*T*), soluble (*S*) and pellet fractions (*P*) were analyzed by SDS-PAGE and immune blotting using anti-hsp60 antiserum. The position of hsp60 is indicated.

identical to those observed with wild type mitochondria (Miller and Cumsky, 1991; see Materials and Methods).

To determine whether sorting of subunit Va was affected in the *mif4* mitochondria, we took advantage of a specific form of subunit Va designated m′ (Miller and Cumsky, 1991). When pVa is imported into mitoplasts (mitochondria with a disrupted outer membrane), or into mitochondria that are subsequently converted to mitoplasts, both mature Va (m) and a faster migrating form (m′) are always observed after proteinase K treatment of the organelles (Fig. 2, lane *4*; Miller and Cumsky, 1991). Fig. 2 shows that when pVa was imported into mitoplasts in which the matrix protease activity was blocked by the presence of *o*-phenanthroline and EDTA, the mature and m′ forms were not formed. Instead, we observed two distinct species corresponding to full-length proteinase K–protected precursor (p), and a new form referred to a p′ (Fig. 2, lane *6*). The p and p′ forms could be "chased" into m and m′ by reactivating the matrix protease with Mn^{2+} (Fig. 2, lane *8*). Thus, m′ represents a form of subunit Va that must span the inner membrane in a manner that leaves the COOH-terminal portion of the protein exposed to the intermembrane space and accessible to externally added proteinase K, while the NH_2 terminus is exposed to the matrix. Thus far, the available data suggest that m′, which is firmly inserted into the bilayer and is not extractable with alkali, is generated from unassembled subunit Va in the mitochondrial inner membrane (Miller, B. R., unpublished results). Nevertheless, m′ is only observed when subunit Va has been correctly sorted to the inner membrane and the integrity of the outer membrane has been disrupted. Derivatives of subunit Va that are mislocalized to the matrix do not generate the m′ species upon protease digestion of mitoplasts (Jung, L. A., B. R. Miller, and M. G. Cumsky, manuscript in preparation).

As shown in Fig. 1 *A*, mitoplasts formed from *mif4* mitochondria were fully competent to efficiently import pVa. When treated with proteinase K, both the wild-type and *mif4* mitoplast preparations also gave rise to the m′ form, indicating that subunit Va had been correctly sorted to the inner membrane. Thus, the results of Fig. 1 *A* demonstrate that pVa

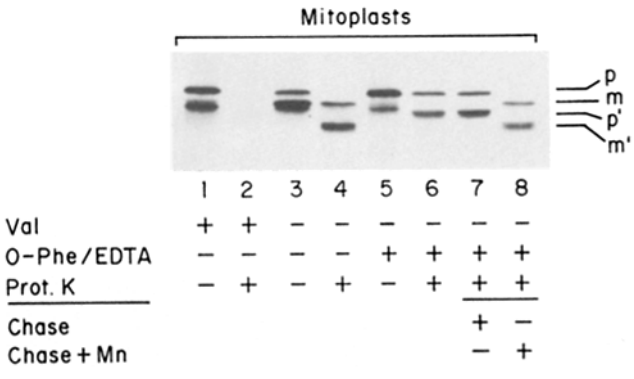

Figure 2. The m′ species spans the inner membrane and is indicative of correct intramitochondrial sorting. Mitochondria (50 μg per reaction) were converted to mitoplasts (Miller and Cumsky, 1991) and resuspended in 100 μl TRB. Mitoplasts were preincubated with 5 μg/ml Val (lanes *1* and *2*), or 1 mM *o*-phenanthroline/10 mM EDTA (lanes *5–8*) for 5 min at room temperature. 15,000 cpm radiolabeled pVa was added to each reaction and the samples incubated at room temperature for 5 min. They were then stopped by the addition of Val (see Materials and Methods). Where indicated (reactions 2, 4, 6, 7, 8) the reactions were digested with proteinase K as described in the legend to Fig. 1 *A*. The mitoplasts from reactions 1–6 were then recovered by centrifugation and processed for SDS-PAGE. Mitoplasts from reactions 7 and 8 were recovered by centrifugation and resuspended into 100 μl TRB containing 5 μg/ml Val and 1 mM *o*-phenanthroline/10 mM EDTA (*Chase*; lane *7*) or 5 μg/ml Val and 10 mM $MnCl_2$ (*Chase + Mn*; lane *8*). The chase reactions were incubated for an additional 10 min at room temperature. The mitoplasts were recovered (above) and processed for SDS-PAGE. All samples were then analyzed by SDS-PAGE and fluorography. The respective positions of pVa (*p*), mature Va (*m*), and the m′ and p′ digestion products (see text) are indicated.

is efficiently imported and correctly sorted to the inner membrane in mitochondria lacking functional hsp60. The results presented in Fig. 2 confirm that the NH_2 terminus of pVa is exposed to the matrix and processed by the matrix metalloprotease.

Several control experiments were performed to demonstrate conclusively that hsp60 was nonfunctional in our preparation of *mif4* mitochondria. First, we examined the import and assembly of another mitochondrial protein, the precursor to the β subunit of the F_1ATPase ($pF_1\beta$). To examine the assembly of $F_1\beta$, we took advantage of the observation that when mitochondria are extracted with chloroform assembled F_1ATPase complexes partition into the aqueous phase, while unassembled subunits partition into the organic phase (Cheng et al., 1989). As shown in Fig. 1 *B*, $pF_1\beta$ was imported into wild-type mitochondria and processed to the mature species (Fig. 1 *B*, lanes *1* and *2*). Upon extraction of the mitochondria with chloroform, a significant fraction (~20–25%) of the mature $F_1\beta$ was recovered in the aqueous phase, suggesting that it had been assembled into an F_1 complex (Fig. 1 *B*, lane *3*). In contrast, the *mif4* mitochondria were severely impaired in their ability to import and correctly process $pF_1\beta$ (Fig. 1 *B*, lanes *5* and *6*). Furthermore, the small amount of $F_1\beta$ that remained associated with the mitochondria after protease treatment was recovered in the organic phase (Fig. 1 *B*, lane *8*). The gross import defect observed with $pF_1\beta$ in these mitochondria is particularly striking when compared to the results observed for pVa, where import efficiency appeared normal with the same preparation of organelles (Fig. 1 *A*). The results also differ somewhat from the less severe import defect previously observed for $pF_1\beta$ in *mif4* mitochondria (Cheng et al., 1989). We attribute the latter difference, which is completely reproducible in our hands, to the conditions under which we perform our experiments. We routinely isolate mitochondria from the α143 strain after a 3.5 h temperature shift. In the previous report, the temperature was shifted for only 1 hour prior to preparation of the organelles (Cheng et al., 1989).

Fig. 1 *C* shows the results of an experiment further testing our *mif4* mitochondrial preparation. It has been demonstrated that after a temperature shift to 37°C hsp60 from *mif4* mitochondria becomes insoluble in the non-ionic detergent Triton X-100, while the wild-type (assembled) form is largely soluble (Cheng et al., 1989). As shown in the figure, we found this to be precisely the case. The vast majority of the hsp60 detected on an immune blot after Triton X-100 extraction of wild-type mitochondria was found in the soluble fraction (Fig. 1 *C*, left three lanes). The hsp60 in the *mif4* mitochondria was completely insoluble under the same conditions and was recovered in the pellet after centrifugation of the Triton extract (Fig. *1* C, right three lanes). Thus, from the combined results of Fig. 1, *B* and *C*, we conclude that hsp60 was nonfunctional under the conditions of our experiments.

It has been reported that hsp60 function in the mitochondrial matrix is sensitive to the membrane-permeable alkylating agent NEM (Ostermann et al., 1989). Thus, NEM pretreatment would be expected to interfere with the import and sorting of precursor proteins whose import pathway contains an hsp60-dependent step. When wild type mitochondria were pretreated with 5 mM NEM, we observed no significant effect on either the import (Fig. 3, lanes *1–4*) or sorting (as judged by the formation of the m′ species, Fig. 3, lanes *5–8*) of pVa. On the other hand, the import of $pF_1\beta$ was completely blocked by the NEM pretreatment (Fig. 3, lanes *9–12*). These results provide additional evidence that pVa can be efficiently imported and correctly sorted to the inner membrane without the aid of a functional hsp60 complex.

Import and Sorting of pVa in Mitochondria Lacking Functional hsp70

The *SSC1* gene is essential for the viability of yeast strains and has been shown to be necessary for the translocation of several mitochondrial precursor proteins (Kang et al., 1990; Ostermann et al., 1990; Scherer et al., 1990; Manning-Krieg et al., 1991). It has also been proposed that the *SSC1* product, mhsp70, participates in driving translocation by binding to partially imported intermediates and "pulling" them into the matrix (Neupert et al., 1990; Ostermann et al., 1990; Scherer et al., 1990; Manning-Krieg et al., 1991). To determine whether mhsp70 was required for the import of pVa we used the yeast strain BC100(*ssc1-2*), which expresses a temperature-sensitive derivative of mhsp70 (Kang et al., 1990). In parallel experiments, mitochondria were isolated from both wild-type and *ssc1* cells. The *ssc1* mitochondria were then shifted to 37°C for 15 min (to inactivate the thermolabile mhsp70) and tested for their ability to import either

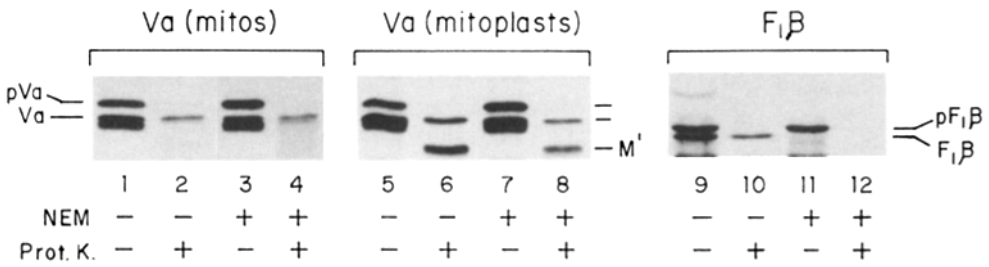

Figure 3. Inactivation of hsp60 by NEM treatment of mitochondria does not block import or sorting of pVa. Mitochondria (lanes *1–4* and *9–12*) or mitoplasts (lanes *5–8*) prepared from wild-type yeast were either pretreated with NEM (lanes *3*, *4*, *7*, *8*, *11*, and *12*) or NEM + DTT (lanes *1*, *2*, *5*, *6*, *9*, and *10*) as described in Materials and Methods. Import reactions were performed as described in the legend to Fig. 1 *A*, except that for $pF_1\beta$, the reactions contained 20,000 cpm of radiolabeled precursor protein. After a 5-min incubation at room temperature the reactions were terminated with Val and where indicated digested with proteinase K. The position of pVa, mature subunit Va (*Va*), the m′ digestion product (*M′*), $pF_1\beta$, and mature $F_1\beta$ (*$F_1\beta$*) are indicated. The band below mature subunit Va, which is not imported and always remains sensitive to proteinase K, results from a nonspecific downstream initiation of the *COX5a* mRNA. It is prevalent with some batches of reticulocyte lysate (see Fig. 2, lane *1*).

pVa or $pF_1\beta$, a control precursor protein whose import has been shown to be dependent upon mhsp70 (Kang et al., 1990; Manning-Krieg et al., 1991). As shown in Fig. 4, significant amounts of both pVa and $pF_1\beta$ were imported to their protease-protected mature forms in wild-type mitochondria (Fig. 4, lanes *1* and *2*). For the *sscl* mitochondria we observed a nearly identical result for pVa: ~70% of the mature protein generated was protected from externally added proteinase K. We also found that import and processing of pVa was dependent on a membrane potential (not shown) and, based upon the formation of the m′ form, that the mature subunit was correctly inserted in the inner membrane (Fig. 5). For $F_1\beta$, essentially no protease-protected mature protein was observed in *sscl* mitochondria (Fig. 4, *right panel*, lanes *3* and *4*). Consistent with previous reports (Kang et al., 1990), we found that $F_1\beta$ accumulated as a contact site intermediate; much of the precursor was processed but remained sensitive to digestion by externally added proteinase K (Fig. 4, *right panel*, lane *4*).

The results presented in Fig. 4 demonstrate that pVa can be efficiently imported and correctly sorted in yeast mitochondria containing an inactive form of mhsp70. However, from this single experiment we cannot rule out the possibility that a small percentage of the mhsp70 molecules in the *sscl* mitochondria remain active after thermal inactivation. We conclude therefore that pVa is imported either without, or at the very least, with a reduced requirement for mhsp70 function in the matrix. While this result was initially surprising given current models suggesting that ATP-driven mhsp70 function facilitates translocation into mitochondria, we suggest that it fits well with several previously published observations from this and other laboratories. First, it has been shown that predenaturation of a precursor protein with urea can alleviate the translocation block caused by a lack of func-

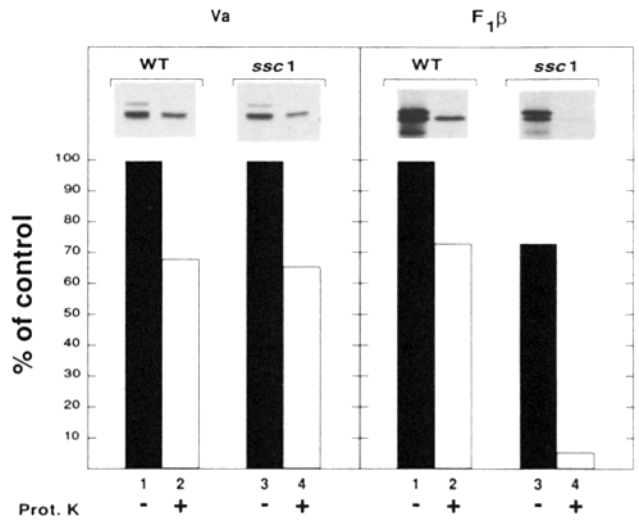

Figure 4. pVa is imported into mitochondria lacking functional hsp70 in the matrix. Mitochondria were prepared from either the wild-type strain or strain BC100(*sscl-2*) as described in Materials and Methods. Import reactions using the wild-type (*WT*; lanes *1* and *2* in each panel) or *sscl* mitochondria (*sscl*; lanes *3* and *4* in each panel) were then performed using 50 μg mitochondria and 15,000 cpm radiolabeled pVa or 20,000 cpm radiolabeled $pF_1\beta$. As described in Materials and Methods, the *sscl* mitochondria were heat shocked for 15 min before use in the import reactions. Reactions were incubated for 5 min at room temperature, terminated with Val, and where indicated, treated with proteinase K (*Prot. K*) as described in Materials and Methods. The protease reactions were terminated with PMSF and the samples processed for and analyzed by SDS-PAGE and fluorography. The resulting fluorographs were quantified by densitometry (columns). The amount of processed protein present in wild-type mitochondria before proteinase K digestion was set to 100%.

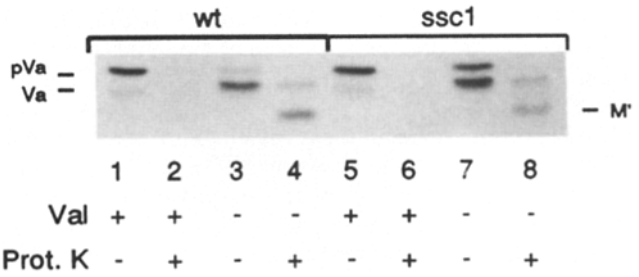

Figure 5. pVa is correctly sorted in mitochondria lacking functional hsp70 in the matrix. Wild-type and *sscl* mitochondria were prepared and converted to mitoplasts as described in Materials and Methods. Heat shock of *sscl* mitochondria and import reactions were as described in the legend to Fig. 4. Val (5 μg/ml) was added to control reactions (lanes *1*, *2*, *5*, and *6*) before the addition of pVa. Proteinase K digestions (as indicated) were performed as described in the legend to Fig. 4. The position of pVa, mature Va (*Va*) and the m′ digestion products (*M′*) are indicated.

tional mhsp70 in the matrix (Kang et al., 1990). Second, our own results have shown that because pVa is loosely folded it behaves essentially like a denatured protein (below). Thus, the results presented in Figs. 4 and 5 do not argue against mhsp70 playing a key role in protein translocation. Rather, they suggest that the unique physical properties of pVa allow it to circumvent an obligate requirement for mhsp70 function in the matrix.

Import and Sorting of pVa in the Absence of High Levels of Cytosolic hsp70 or NEM-sensitive Factors

Our previous results suggested that pVa is not tightly folded in solution (Miller and Cumsky, 1991). Included among these are the findings that pVa is extremely sensitive to proteolytic degradation, that it is imported with unusual efficiency even at very low temperature, and that its import efficiency is not enhanced by prior denaturation of the protein. We have also shown that protease-sensitive surface receptors are not required for efficient import and sorting of pVa (Miller and Cumsky, 1991). It was therefore of interest to determine whether cytosolic hsp70 (chsp70) or other soluble factors were required for the import of pVa into yeast mitochondria.

The loss of all chsp70 function is lethal in *S. cerevisiae*, as strains carrying mutations or disruptions in four genes specifying isoforms of chsp70 (*SSA1-SSA4*) are nonviable (Deshaies et al., 1988). The lethality can be conditionally suppressed, however, by introducing an extrachromosomal copy of one of the genes (*SSA1*) under the control of an inducible promoter (Deshaies et al., 1988). Such yeast strains are viable only when the *SSA1* gene is induced, as a shift from inducing to noninducing conditions causes depletion of the cellular pool of chsp70. Using this strain, Deshaies et al. (1988) demonstrated that as chsp70 becomes limiting (shifting to noninducing conditions) precursors for both a secreted and an imported mitochondrial protein accumulate (prepro-α-factor and $pF_1\beta$, respectively). When we performed an identical experiment under the same conditions, we detected the accumulation of precursor forms of both $F_1\beta$ and hsp60 (data not shown). However, we saw no accumulation of pVa. Instead, we observed significant amounts of mature subunit Va that gradually decreased as the cells stopped growing and the chsp70 was depleted (data not shown). These results are consistent with the interpretation that pVa is imported in the absence of chsp70. However, we cannot exclude the more trivial possibility that the subunit Va precursor is unstable outside mitochondria in vivo.

To address further the role of chsp70 on the import of pVa, the precursor was generated in a wheat germ–based translational extract. It has been shown that the wheat germ cell-free system does not contain sufficient amounts of at least two cytosolic components necessary for the import of precursor proteins into mitochondria (Murakami et al., 1988). The first of these is chsp70, and the second is one or more factors sensitive to NEM.

As shown in Fig. 6, pVa translated in the wheat germ system was imported into isolated yeast mitochondria in a potential-dependent fashion (Fig. 6, lanes *1–4*). We also found that it was correctly sorted to the inner membrane (data not shown). To ensure that the import assay was performing faithfully with these extracts, we again studied the

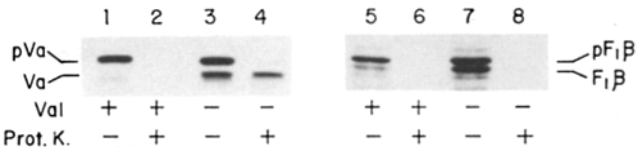

Figure 6. pVa is imported into mitochondria in the absence of high levels of chsp70. In vitro import reactions were performed using 50 μg mitochondria and 15,000 cpm radiolabeled pVa or 20,000 cpm radiolabeled $pF_1\beta$ (generated in a wheat germ cell-free translation system) as described in Materials and Methods. Valinomycin (*Val*; 5 μg/ml) was added to control reactions (lanes *1*, *2*, *5*, and *6*). Proteinase K digestions (as indicated) were performed as described in the legend to Fig. 4. The position of pVa, mature subunit Va (*Va*), $pF_1\beta$ and mature $F_1\beta$ (*$F_1\beta$*) are indicated.

import of $pF_1\beta$. In contrast to the results for pVa, protease-resistant mature $F_1\beta$ was not observed when mitochondria were incubated with precursor translated in the wheat germ extract (Fig. 6, lanes *7* and *8*). Interestingly, and as we had observed earlier when we analyzed import into *ssc1* mitochondria (Fig. 4), the import of $F_1\beta$ was arrested at contact sites (Fig. 6, lane *7*). Thus, the results of Fig. 6 demonstrate

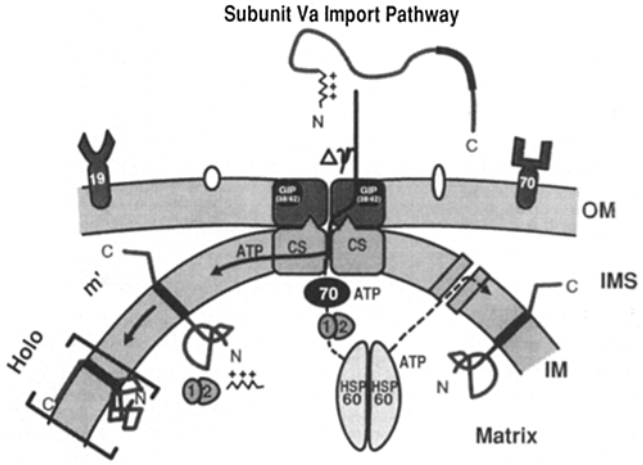

Figure 7. Working model of the subunit Va import pathway. pVa is drawn as loosely folded and achieves import competence without the aid of soluble factors; the presequence is shown as the positively charged region at the NH_2 terminus and the sorting sequence (IMSS) is shown as the bold region near the COOH terminus. The established pathway is indicated by the solid arrow. In the presence of a membrane potential ($\Delta\psi$) pVa interacts directly with the import site in the outer membrane without using surface receptors. Translocation of the presequence into the matrix is $\Delta\psi$ dependent, and occurs through contact sites. The IMSS arrests translocation at the inner membrane and prevents release of the protein. The presequence is removed by the matrix metalloprotease (proteins labeled 1 and 2 represent the subunits of the protease) during or after translocation. After subunit Va partitions from the contact sites it is assembled into the oxidase complex (*brackets*). The requirement for ATP at the inner membrane is hypothetical; the conservative sorting pathway shown using the dashed line is for schematic purposes, as under our conditions it does not appear to be used. *19*, import receptor MOM19; *70* (outer membrane), import receptor MAS70; *CS*, contact sites; *GIP*, general insertion pore containing MOM38 (*N. crassa*) or ISP42 (yeast); *70* (matrix), mitochondrial hsp70; *m'*, subunit Va species that gives rise to the m' intermediate; *Holo*, cytochrome *c* oxidase holoenzyme.

that while significant levels of hsp70 and one or more factor(s) sensitive to NEM are required for the complete import of $pF_1\beta$, pVa can be imported without them. The results also support our earlier contention that ATP hydrolysis outside mitochondria is not required for the import of pVa (Miller and Cumsky, 1991).

Discussion

This study extends our earlier work on the import pathway of the precursor to subunit Va of yeast cytochrome *c* oxidase. We show here that pVa can be efficiently imported and correctly sorted to the mitochondrial inner membrane in the absence of a functional hsp60 complex. We also show that the import and sorting of pVa can occur in the absence of a functional hsp60 complex. We also show that the import and sorting of pVa can occur in the absence of high levels of functional mitochondrial and cytosolic hsp70, findings that we attribute, at least in part, to the unusual physical properties of the subunit Va precursor. In Fig. 7 we present our working model of the subunit Va import pathway.

The loosely folded Va precursor associates with the mitochondrial surface through an interaction that is not mediated by protease-sensitive receptors; pVa is then imported through contact sites in a potential-dependent fashion (Miller and Cumsky, 1991). Although the possibility that pVa follows more than one sorting pathway cannot be rigorously excluded (*dashed line* in Fig. 7), we propose that under the conditions of our experiments the precursor is delivered to the inner membrane via a pathway that does not involve complete translocation into the matrix (nonconservative sorting). Several lines of experimental evidence strongly support this view. The most compelling are the results of this study demonstrating that hsp60 function is not required for import and sorting of pVa. The conservative sorting model predicts, and several published reports show, that hsp60 function is essential for the reexport of proteins that are routed through the matrix (Cheng et al., 1989; Ostermann et al., 1989; Mahlke et al., 1990). The rapid import and sorting kinetics of pVa at 0°C, and our failure to identify a subunit Va/hsp60 complex in ATP-depleted mitochondria, also argue against a matrix-localized intermediate (see Results; Miller and Cumsky, 1991).

Our work on the subunit Va sorting signal (IMSS) further supports the sorting model presented in Fig. 7. The subunit Va IMSS is a membrane-spanning domain in the COOH-terminal third of the protein (Glaser et al., 1990). Recent results have shown that when attached to the COOH terminus of a matrix protein (an artificial precursor protein containing the matrix targeting portion of the cytochrome c_1 presequence fused to mouse dihydrofolate reductase, DHFR), the IMSS redirected the protein (referred to as pc_1DHFR-Va) to the inner membrane (Jung, L. A., B. R. Miller, and M. G. Cumsky, manuscript in preparation). Importantly, when imported into mitoplasts and treated with proteinase K, the pc_1DHFR-Va fusion gave rise to an m'-like species and was therefore oriented in the membrane precisely as native subunit Va (Fig. 2; Jung L., B. R. Miller, and M. G. Cumsky, manuscript in preparation). When taken together, the results suggest that the hydrophobic IMSS functions as stop-transfer or membrane anchor sequence, and not as a reexport signal. The known reexport signals, which reside close to the NH_2 terminus and correspond to the hydrophobic portions of bipartite presequences (Hartl et al., 1987), have been shown to function in part by inhibiting hsp60-mediated folding of precursors in the matrix (Koll et al., 1992). It seems unlikely that the IMSS could function in this manner, as it is located approximately 100 amino acids from the NH_2 terminus of pVa and ~220 amino acids from the NH_2 terminus of pc_1DHFR-Va.

While ATP hydrolysis within mitochondria is required for the import of pVa, the levels of ATP required are lower than those needed for the import of $pF_1\beta$ (Miller and Cumsky, 1991). In light of our finding that pVa can be imported in the absence of functional hsp60, the question of which step on the subunit Va import pathway requires ATP hydrolysis arises. At present, the answer to this question remains to be determined. However, several possibilities exist. First, it is possible that an unidentified chaperone-like protein may participate in the sorting and/or assembly of subunit Va. Such a protein might function at the level of the inner membrane as has been proposed for the products of the *COXI0* and *COXI1* genes (Nobrega et al., 1990; Tzagoloff et al., 1990). Alternatively, and as discussed in Results, our data concerning import into *sscl* mitochondria does not eliminate the possibility that mhsp70 plays a distinct, albeit diminished, role in the import of pVa. Since it is clear that the NH_2 terminus of pVa is exposed to the matrix (Fig. 2), it remains possible that at least part of the ATP requirement observed for pVa may be attributable to mhsp70 function in wild-type mitochondria. The reduced demand for mhsp70 in pVa import (Figs. 4 and 5), may in turn reflect the fact that only ~70 amino acids of the Va polypeptide need to be "pulled" into or across the inner membrane before the IMSS blocks further transfer of the protein. It should be noted, however, that while we assume the NH_2-terminal portion of mature subunit Va is transported into the matrix (Fig. 7), we have no direct evidence that this is the case. It is possible that only the presequence is significantly exposed to the matrix and that the rest of the protein remains tightly associated with the inner membrane.

The results of this study establish a new sorting pathway for integral inner membrane proteins containing NH_2-terminal presequences. This pathway uses the translocation contact sites formed from the import channels of the outer and inner mitochondrial membranes and requires at least one activity located in the matrix (the processing metalloprotease), but it does not use hsp60. It appears to be distinct from either the conservative pathway followed by other inner membrane proteins (e.g., $pF_1\beta$ and the precursor to the F_0 portion of the *N. crassa* ATPase; Cheng et al., 1989; Mahlke et al., 1990), or the nonconservative pathway followed by the precursor to AAC (Mahlke et al., 1990). Our future studies will be focused on several of the questions concerning the import of pVa that remain unanswered. We are especially interested in more precisely analyzing the role of ATP hydrolysis in pVa import, and determining whether the NH_2 terminus of the mature protein is transported to the matrix. We are also interested in exploring the function of the subunit Va IMSS. The results of those studies will hopefully enhance our overall understanding of both mitochondrial import and protein translocation into and across all biological membranes.

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